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Survival of *Enterococcus faecium* in Turkey Litter Under Different Temperature and Moisture Combinations

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ABSTRACT

Untreated poultry litter introduces a substantial load of fecal pathogens to the environment, impacting agriculture and ecosystem function. There is substantial evidence that temperature and moisture are the primary drivers of fecal bacteria survival across ecosystems. However, both temperature and moisture effects have been shown to be modulated by the matrix in which the fecal bacteria are living. This context dependence highlights the importance of understanding fecal bacteria survival in a variety of matrices in order to implement effective waste management plans. In this study, the survival patterns of *Enterococcus faecium* in post-use turkey litter under two levels of temperature (5°C, 30°C) and moisture (<10% w/v, ~35% w/v) were determined. Under both moisture conditions at 5°C, *E. faecium* abundance did not change over the course of 14 days. However, at 30°C, the low moisture treatment resulted in 23% decrease in *E. faecium* concentration over 14 days, while the high moisture treatment resulted in 16% growth over the course of the experiment. Since high temperature is usually sufficient to result in fecal bacteria decay in other matrices, this study highlights the context dependence of fecal bacteria survival. These findings support current manure management recommendations to apply litter during warm months, when soil is not wet.

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INTRODUCTION

Each year, Virginia poultry farms produce millions of kilograms of poultry waste (Pelletier et al. 2001) which contain high concentrations of fecal pathogens (Kelley et al. 1995; Martin et al. 1998; Terzich et al. 2000). Poultry litter, a combination of excreta, bedding, feathers, and feed, is a cost-effective fertilizer (Bosch and Napit 1992), which can be used to produce higher crop yields due to its excess nitrogen (N) and phosphorus (P) content (Wood et al. 1993; Evers 1998; Nyamasoka et al. 2017). However, livestock wastes have also been shown to contaminate crops (Heaton and Jones 2008; Atidégla et al. 2016; Pizarro et al. 2019) and freshwaters (Haack et al. 2016), as well as impacting ecosystem functioning (McBride et al. 2020; Wepking et al. 2017). In order to reduce the negative effects of fecal contamination and protect Virginia's natural resources and agricultural production, it is important to understand the dynamics of fecal bacteria survival in poultry litter.

In terrestrial environments, temperature and moisture have long been known to drive survival of fecal bacteria (Kibbey et al. 1978; Zibilske and Weaver 1978; Reddy et al. 1981), including pathogens (Zibilske and Weaver 1978; Cools et al. 2001). Despite the fact that high temperatures are related to increased decay of fecal bacteria (Blaustein et al. 2013), temperature was found to be the best predictor of their presence in a watershed (Badgley et al. 2019). Dry soil is generally unfavorable for the survival of fecal bacteria (Donsel et al. 1967; Pepper et al. 1993; Staley et al. 2016). It is possible, however, for these organisms to regrow once soil is moistened (Chandler and Craven 1980; Hartel et al. 2005). While it is clear that temperature and moisture drive fecal bacteria's survival in soil and water, the effect of these environmental variables in poultry litter remains unclear. Indeed, the survival of fecal bacteria can be modulated by matrix-specific characteristics. For example, diurnal temperature oscillations were shown not to affect the survival of fecal bacteria in soils with high clay content but were important in soils with high sand content (Smith et al. 2019). Likewise, saturated sand at beaches with smaller grains supported fecal bacteria for longer periods than saturated soil with larger grain size (Staley et al. 2016). These matrix-specific effects highlight the importance of understanding the survival dynamics of fecal bacteria in diverse environments.

Previous studies have investigated survival of fecal bacteria after poultry litter has been applied to land (Gu et al. 2019; Neher et al. 2019), as well as the effect of different temperature dependent treatments on removing fecal bacteria from stored poultry litter (Ngodigha and Owen, 2009; Wilkinson et al. 2011). Treatment of poultry litter is not always completely successful (Hartel et al. 2005; Neher et al. 2015), and animal manure is not always required to be treated before application as a fertilizer (USDA 2015a). Therefore, when poultry litter is applied in agricultural settings, fecal organisms enter the ecosystem in addition to the desired nutrients.

This study aims to determine how temperature and moisture affect the survival of the fecal indicator bacterium (FIB) *Enterococcus faecium* in turkey litter. Turkey litter is of particular importance to Virginia since the state is the 6th highest producer of turkeys in the United States (USDA 2020), and survival of fecal organisms does not appear to have been tested in this matrix, even though it is commonly used as fertilizer. In order to determine the effects of temperature and moisture on *E. faecium* survival we used a full factorial laboratory experiment with two levels of temperature, and two levels of moisture. We predicted that *E. faecium* decay would be facilitated

by both high temperature and low moisture and that survival would be promoted by low temperature and high moisture conditions.

METHODS

Study Site and Sample Collection

All turkey bedding and litter samples were acquired from a poultry house on a farm in Shenandoah County, VA. The litter was composed of hardwood shavings, a common material, that were reused between each flock for three successive flocks of 7,000 to 9,000 turkeys each. In between each flock the litter was windrow-composted, and each windrow was turned approximately every 72 hours for a total of three turns. Six individual composite samples were collected by combining six grab samples from the top five cm of bedding, homogenizing them, and storing at 4°C during transport to the laboratory. The gravimetric water content was determined by drying 10 g of litter from each composite sample at 105°C for 24 h.

Study Organism Description

Enterococci were isolated from individual composite samples using EPA Method 1600 (US EPA, 2014) and modified for use with poultry litter. Briefly, the contents of the microcosm were added to 500 mL of PBS in a 1 L shaker flask, and bacteria were dispersed by shaking for one hour using a Burrell Wrist Action Shaker™ (75-775-24; Model 75, Pittsburgh, PA) (Zuberer 1994), then allowed to sit for 30 minutes to allow debris to settle out of solution. While it is likely that some of the suspended bacteria also settled during this time it should have done so at a similar rate between treatments. Supernatant was serially diluted and filtered onto a 0.45 µm nitrocellulose filter, which was placed on membrane-Enterococcus Indoxyl-β-D-Glucoside (mEI) Agar (215047; Becton, Dickinson and Company, Sparks, MD) and allowed to incubate 24 h at 41°C, before being counted. Putative enterococci presented a blue hue. A subset of blue isolates was selected from plates and identified to species using the Biolog System, following the manufacturer's instructions (71102; Biolog, Inc., Hayward, CA).

Enterococcus faecium was identified as the most common species isolated from these samples (data not reported), and American Type Culture Collection 19434 was used in all of the laboratory experiments. *Enterococcus faecium* is a fecal indicator bacterium (FIB) and is recommended as a proxy for pathogens by the United States Environmental Protection Agency for detecting contaminated water ways (US EPA 2012, 1986, 1976). Enterococci are Gram-positive, catalase-negative, facultative anaerobic cocci, which can grow at temperatures between 10 and 45°C, in broth containing 6.5% NaCl, and at pH 9.6. They are also able to hydrolyze esculin with the presence of bile salts, and they present the Lancefield group D antigen (Facklam et al. 2002). The wide range of environmental tolerances and their commensal presence in the poultry gut microbiota make the enterococci an ideal model system for studying the survival of fecal bacteria in poultry litter.

Microcosm Experiments

A 2 x 2 factorial experiment was conducted with two levels of temperature (5°C and 30°C) and two levels of moisture (<10% and 35% w/v water); hereto the treatments will be referred to as

'5°C/Dry', '5°C/Wet', '30°C/Dry', and '30°C/Wet'. The two temperatures were used to approximate mean temperatures in Shenandoah County, VA during the summer and winter, while the two moistures were chosen based on accounts that stored litter often has a dry outside layer while deeper in the pile there is higher moisture (Hartel et al. 2000). The experiment was performed twice.

Litter was homogenized after passing through a sieve (2 cm) and sterilized in an autoclave at 121°C for 15 minutes. We used sterilized litter to eliminate ecological interaction, such as predation, and competition with other organisms. Sterile litter was analyzed for nutrient content by The Pennsylvania State University Agricultural Analytical Service Laboratory using the methods listed in Table 1: total N using combustion analysis (Watson et al. 2003); NH_4-N using a specific ion electrode (Peters et al. 2003); P_2O_5 and K_2O using Microwave-assisted acid digestion with inductively coupled plasma spectroscopy (Wolf et al. 2003); Organic N by subtracting NH_4-N from total N.

TABLE 1: Microbiological and nutrient content of litter: including total enterococci (CFU g^{-1} dry litter); total nitrogen (N), Ammonium (NH_4-N), Organic N, phosphorus pentoxide (P_2O_5), and potassium oxide (K_2O); g kg^{-1}.

Enterococcus	**Total N**	**NH_4-N**	**Organic N**	**P_2O_5**	**K_2O**
1.39 x 10^5	47.3	2.9	44.4	42.0	37.5

Individual microcosms were prepared by adding ~10 g of sterile dry weight equivalent turkey litter to a 50 mL conical tube, adjusting to the appropriate moisture content, and inoculating with 1 mL of *E. faecium* inoculum (approximately 1.5 x 10^8 CFU mL^{-1} washed and suspended in PBS). At each time point (+1 hour, 1 day, 7 days, and 14 days) three replicate samples from each treatment were extracted in PBS following the above reported modified EPA Method 1600. For each treatment, controls were prepared by constructing five additional microcosms without the addition of inoculum, and one of these controls was harvested at each time point to verify sterility of the experiments. Throughout the experiment the uninoculated controls were below the colony counting limit. Colony forming units (CFU) were identified as blue colonies on each plate when viewed under magnification. The total CFU g^{-1} dry litter was calculated as the counted blue colonies per the plated volume multiplied by the dilution factor divided by the mass of dry litter. The litter-PBS mixture produced a significant number of suspended particles which made filtering difficult without first performing a 10^{-2} dilution; therefore, the lower counting threshold was 1.2 CFU g^{-1} dry litter.

Statistical Analyses

The total colony forming units (CFU) from the duplicate experiments were $\log_{10}$ transformed and analyzed together with a linear mixed effect model using the lme4 package (Bates et al. 2015) in R version 3.6.1 (R Core Development Team 2019). Some individual replicates were contaminated and were removed from analysis. At each time point, each treatment is represented

by four to six replicates. Temperature, moisture, and incubation time were main effects, and experiment ordinality was a random effect. The model was evaluated for normality of residuals using the shapiro.test command and the residuals were not normally distributed. To account for this, we then performed a general linear mixed effect model fitting the model to a gamma distribution and using the log-link function. We evaluated interactions between moisture and temperature by performing pairwise comparisons within each time point using the TukeyHSD method. We then evaluated the effect of incubation time by performing within treatment pairwise comparisons between the starting concentration of *E. faecium* and the measured concentration at each time point using Dunnett's test, in the *emmeans* package (Lenth et al. 2020); $\alpha = 0.05$.

RESULTS

Microcosm experiments showed a significant main effect of moisture ($P < 0.001$), temperature ($P = 0.002$), and incubation time ($P = 0.027$). There was also a significant 2-way interaction between moisture and temperature ($P < 0.001$) and moisture and incubation time ($P < 0.001$), but not between temperature and incubation time ($P = 0.76$). Finally, there was a significant three-way interaction between all main effects ($P < 0.001$).

When comparing treatments within time points, there was no significant pairwise differences ($P > 0.05$) between the starting concentrations of enterococci, which ranged from $\log_{10}$ 7.1 to 7.6 CFU g^{-1} dry litter. By Day 1, the concentration of *E. faecium* in the 30°C/ Dry treatment declined below the other treatments by 9-13% (all $P < 0.05$). On Day 7, 30°C/Dry treatment continued to contain the lowest concentration of *E. faecium*, which reached 6.1 $\log_{10}$ (CFU g^{-1} dry litter) -- 17-28% less than the other treatments (all $P < 0.001$). While decay continued to occur in the 30°C/Dry treatment on Day 7, the abrupt increase in the percent difference was largely driven by a concomitant increase in *E. faecium* concentration in the 30°C/Wet treatment. At the end of the experiment on Day 14, the 30°C/Dry treatment continued to decline reaching 5.5 $\log_{10}$ (CFU g^{-1} dry litter), 24-38% (all $P < 0.001$), and the 30°C/Wet was 17-19% greater than the two 5°C treatments (both $P < 0.001$).

We also evaluated the difference in enterococci concentration within each treatment over time to determine if there were significant changes from the starting concentration within that treatment. At 5°C, *E. faecium* levels did not change between any of the four sampling days in either the low moisture or high moisture treatments. However, at 30°C both the low moisture and high moisture treatments enterococci concentrations did change with time. At 30°C/Wet, *E. faecium* concentrations increased by 16% by the end of the experiment ($P < 0.001$), a 1.2 $\log_{10}$ CFU g^{1} dry litter increase. In the low moisture experiment, *E. faecium* concentration decreased 14% from Day 0 to Day 7 ($P = 0.007$), and 23% between Day 0 and Day 14 ($P < 0.001$), a ~1.6 log10 CFU g^{1} dry litter decrease over the course of the experiment.

DISCUSSION

Both temperature and moisture affect the survival of *E. faecium* in turkey litter. Specifically, at 30°C, *E. faecium* grow under wet conditions and decay under dry conditions (Figure 1). While we predicted that *E. faecium* would decay most rapidly in warm and dry conditions we did not necessarily expect growth under warm and moist conditions. Since other

studies have found that temperature alone can cause microbial decay (Cools et al. 2001), this study highlights the importance of understanding population dynamics of fecal bacteria in different matrices. Poultry litter may favor growth more than soil alone due to increased availability of carbon and nitrogen for building microbial biomass (Acosta-Martínez and Harmel 2006).

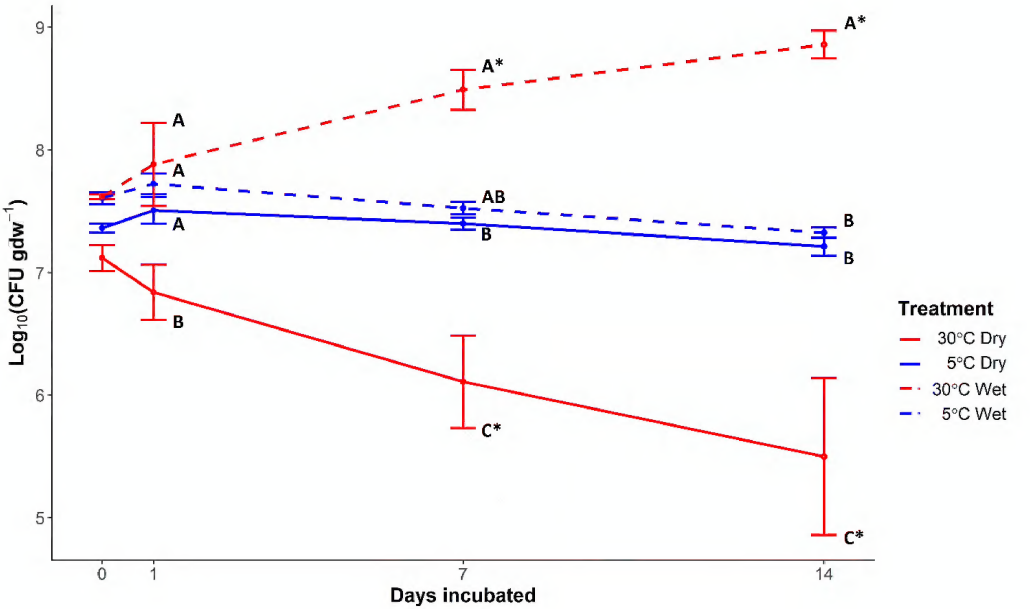

Figure 1: Time series illustrating the survival of *Enterococcus faecium* in turkey litter under different moisture and temperature combinations. Significant treatment differences within an individual time point are denoted by different letters (α=0.10). Significant differences from the starting concentration within a treatment are denoted by an asterisk (α=0.10). Shown are means ± 1 standard error for each time point.

Fecal bacteria in poultry litter have been shown to decay more rapidly when mixed with soil, but to persist when litter was not tilled into the soil (Hruby et al. 2016). It is unclear whether this is due to soil biological or edaphic properties. Due to the increased call for conservation agriculture, such as no till practices (Kassam et al. 2019), it is clear that reducing pathogen load before applying poultry manure as fertilizer is important for ecosystem function. This can be achieved through a variety of composting methods such as windrowing or deep stacking (Ngodigha and Owen 2009; Wilkinson et al. 2011). However, it can be difficult to achieve lethal temperatures throughout the entire compost pile (Tiquia and Tam 2002; Neher et al. 2015), and pathogen regrowth can sometimes occur when environmental conditions improve (Sinton et al. 2007). Additionally, since the demand for fertilizers such as poultry litter decreases during the winter, it is often stored under cooler temperatures, which could lead to longer persistence, as the

concentration of *E. faecium* did not change throughout the duration of this experiment, in either wet or dry conditions at 5°C (Figure 1). Although, this study did not mimic freeze-thaw cycling, which can increase decay of fecal bacteria (Dilek Sanin et al. 1994), the results suggest that there is negligible change in *E. faecium* at low temperature regardless of moisture (Figure 1).

This study provides information that poultry farm managers and regulators can use to determine best practices for land application of poultry litter in order to minimize bacterial impacts on the ecosystem. Future studies should continue to explore environmental and ecological factors that influence survival of enterococci in other environments that mimic field conditions as has been performed in other ecosystems, e.g., use of refugia (Badgley et al. 2010a, 2010b), or competition and predation (Wanjugi and Harwood 2013). Current USDA recommendations (USDA 2015b) for poultry litter application do not explicitly consider survival of pathogens; however, this study supports current recommendations. Poultry litter should not be applied to fields that are wet, or before a precipitation event to reduce the chances of increased moisture leading to a bloom of fecal bacteria. In addition, poultry litter should not be applied under extended cool periods since this could lead to persistence of fecal bacteria in the litter material.

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Summer Food Habits of *Myotis leibii* in the Central Appalachians Ecoregion and Comparison to Similar Studies

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ABSTRACT

Food habits of *Myotis leibii*, Eastern Small-footed Myotis, were studied during summer in the Central Appalachians Ecoregion. Moths were 70.0% of the diet by volume and were in 97.7% of samples (percent frequency). Beetles, flies, and spiders comprised much of the remaining diet. Percent volume and percent frequency metrics produce similar results. These data and past studies indicate this bat eats a relatively low-diversity diet centered on terrestrial-based arthropod prey across a broad geographical area, irrespective of season, sex, or age. The presence of spiders in the diet may indicate gleaning.

INTRODUCTION

The range of *Myotis leibii* (Eastern Small-footed Myotis), extends from northern New England through New York, south along the Appalachian Mountains to North Carolina and westward through Tennessee and northern Georgia, Alabama, and Mississippi (Whitaker and Hamilton 1998). Throughout its wide distribution, it has typically been considered uncommon (Barbour and Davis 1969; Best and Jennings 1997; Johnson et al. 2011, 2012) and the advent of the fungal disease white-nose syndrome (WNS) has further reduced both winter (Frick et al. 2015; Powers et al. 2015) and summer (Francl et al. 2012; Moosman et al. 2013) populations. Food habit studies are limited to sample sizes of 4 (McDowell-Griffith 1983), 39 (Moosman et al. 2007), 44

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go hand-in-hand. *Myotis leibii* forages more in deciduous forests than in large open areas, often near ridge tops (Johnson et al. 2009) where they roost in rock outcrops (Johnson et al. 2011).

The three previous food habits studies all identified moths (order Lepidoptera) as the most abundant diet component by both percent volume and percent frequency, while beetles (order Coleoptera) and flies (order Diptera) ranked second and third in both measures but not always in the same order of abundance across studies. Within studies, ranking between percent volume and percent frequency sometimes differed. Ants (order Hymenoptera), lacewings (order Neuroptera), booklice (order Psocoptera), and leafhoppers (order Homoptera) were ranked fourth or fifth most abundant in at least one study by either percent volume or percent frequency. Spiders were part of the diet in two of the three studies, and while Moosman et al. (2007) ranked them fourth in percent volume and fifth in percent frequency, Johnson et al. (2012) ranked them last (of eight) using both metrics.

Our paper adds to the knowledge of food habits of *M. leibii* from part of the Central Appalachians Ecoregion (Omernik and Griffith 2014) where Virginia, West Virginia, and Kentucky meet. We compare findings, expressed as percent volume and percent frequency of taxa of arthropod prey in the diet, with similar studies. Percent volume is widely used to determine proportions of arthropod taxa in the diet of a group, whereas percent frequency is less widely used to determine how broadly taxa are consumed by individuals of that group (Whitaker 1988). We use a ranking system to compare percent volume and percent frequency across all taxa, for this and previous studies, whereas this comparison is typically limited to individual taxa. This comparison furthers understanding of how the two metrics of diet relate. We use an index of diet diversity as a simple indication of a specialized or generalized diet to compare among our study sites, and among this and previous studies.

MATERIALS AND METHODS

Study Area

Studies were completed in the Central Appalachians ecoregion (Omernik and Griffith 2014), or Allegheny Plateau Physiographic Province, where Virginia, West Virginia, and Kentucky meet. Geologic strata are sedimentary, and the terrain is characterized by high elevation, high relief, and a well-dissected dendritic drainage. The region's naturally rugged terrain is enhanced by extensive bituminous coal mining (surface and sub-surface). While cliff faces and talus rock slopes provide natural roosting habitat for *M. leibii* (Johnson et al. 2011), old above ground mine works such as high walls, and old subsurface mines provide cracks and fissures for roosting. The area has a continental climate with warm to hot summers and cold winters. This is part of Braun's (1950) mixed mesophytic forest region while Dyer (2006) includes it in the Appalachian Oak Section of the Mesophytic Forest.

Field Studies

Bats were captured 15 May to 15 August 2000 and 2001, in Wise County in western Virginia and adjacent counties in southern West Virginia and eastern Kentucky. Mist nets were placed in concave (open) corridors such as roads and utility rights-of-way through upland woodlands, typically with a mature oak-hickory component. In high relief terrain, pools of drinking water are uncommon, so nets were often placed near water-filled road ruts (Kiser and MacGregor 2004; Johnson et al. 2011, 2012); reproductive female *M. leibii* often roost close to water sources (Johnson et al. 2011). Netting followed protocol of the U.S. Fish and Wildlife Service for the endangered *Myotis sodalis* (Indiana Myotis) at that time (current version at USFWS 2020). The protocol required mist nets extend from ground to canopy, usually requiring two to three vertical tiers of nets and two nights of netting with two nets sets (four net nights) for each 100 ha of suitable habitat sampled.

When bats were removed from the net, morphometric measurements, sex, age, and reproductive condition were recorded. *M. leibii* were placed in individual cloth bags for 10–20 minutes to collect fecal samples. Feces were placed in catalogued containers, air-dried, and transported to the laboratory. Bat capture and handling followed guidelines of the American Society of Mammalogists (Animal Care and Use Committee 1998).

Fecal Analyses

Guano samples were obtained from 43 bats: 23 from western Virginia, 15 from eastern Kentucky, and 5 from southern West Virginia (Table 1). In the laboratory, pellets from a single bat were placed on a petri dish and wetted with an ethanol and water mixture and teased apart. Insect remains were identified using a dissecting microscope, and percent volume was estimated visually for each arthropod taxon (insects and spiders). Insects were identified to the lowest taxon possible, usually order and sometimes family. The volume of remains of each taxon in the diet of a bat was expressed as a percent of that bat's total diet. When diets of individuals were combined, guano from each bat was treated as a single unit regardless of the number of pellets, so each bat contributed equally to the combined group diet, avoiding bias from samples weighted by numbers of pellets. Data were summarized by percent volume (volume of each food/total volume x 100) and percent frequency, the percentage of bats in a sample eating arthropods of a particular taxon (Whitaker 1988).

For each sample (by state and combined), a diet diversity index (DDI) was calculated: $DDI = 1/\sum P_i^2$ (MacArthur 1972), where P_i is the proportional occurrence of an arthropod taxon (seven insect orders plus spiders) in a sample. This index is a quick estimate of the number of equally represented taxa in the diet, which provides an easy indication of a specialized or generalized diet but does not identify taxa.

Bats of all ages and sexes were pooled by state because of small sample sizes. Analysis of variance (ANOVA) was used to compare arthropod taxa among pooled state samples. Analyses of percent volume uses arcsine-transformed data to stabilize variance and normalize proportional data in biological studies (Sokal and Rolf 1981) and is frequently employed in bat dietary analyses

(Humphrey et al. 1983; Brack and LaVal 1985, 2006; Dalton et al. 1986; Whitaker 1988; Sample and Whitmore 1993; Brack and Whitaker 2001; Moosman et al. 2007; Rakotoarivelo et al. 2007).

Percent volume and percent frequency are frequently used metrics of bat diets. Although often compared within individual taxon, there is no standard comparison across the many taxa that comprise a diet. We made this comparison by ranking taxa by percent volume and percent frequency, most to least common, and subtracting the rank for percent frequency from rank for percent volume. This produced a zero, positive, or negative number, indicating ranks were the same, or that percent frequency ranked lower or higher than percent volume, with numerals indicating the number of levels of rank change.

Comparison to Similar Studies

We compared the combined sample for the Central Appalachians Ecoregion to similarly pooled samples of previous studies (Johnson and Gates 2007; Moosman et al. 2007; Johnson et al. 2012). Comparisons include (1) ANOVAs on percent volume and percent frequency of all arthropod taxa, (2) ANOVAs on the four most common taxa (moths, beetles, flies, and spiders) because DDI in each study was between four and five, (3) percent volume versus percent frequency, and (4) DDIs. We graphically display (compare) percent volume and percent frequency of the four most common taxa from this and previous studies.

RESULTS

Food Habits in the Central Appalachians Ecoregion

We identified arthropod remains in feces to seven orders of insects plus spiders; some insect remains were identified to 10 families. Moths, the most important food (Table 1), were in the diet of all but one individual bat. Beetles were second most important in the diet. Remains of scarab beetles (family Scarabaeidae) were in five samples from Virginia, four from Kentucky, and one from West Virginia, while remains of leaf beetles (family Chrysomelidae) were in two samples from Virginia and one sample from Kentucky. Flies were the third most common food; remains of crane flies (family Tipulidae) were in feces of two bats from Virginia. Leafhoppers (family Cicadellidae, order Homoptera) were a relatively small part of the diet by volume but were in samples from all three states and 34.9% of all samples. Spiders were in samples from each state and 18 of 43 bats (Table 1).

Location (state) had no significant impact on percent volume ($F_{(2,24)} = .068$, $p = .934$) or percent frequency ($F_{(2,24)} = .014$, $p = .985$) of arthropod taxonomic groups in the diets. As such, data were pooled across the ecoregion (Table 1). The DDI was 5.06 for the pooled sample and 4.57 to 5.20 within states (Table 1). Rankings of taxa in the diet, most to least common, were similar for percent volume and percent frequency (Table 2). In individual states, only three taxa varied by more than one rank (Table 2), and when combined, the rank of two taxa each varied by a single rank.

TABLE 1. Foods (arthropod taxa) of *M. leibii* from the Central Appalachians Ecoregion by percent volume (Vol.), percent frequency (Freq.), and number of occurrences in the sample (No.). The Diet Diversity Index (DDI) provides an estimate of the number of equally represented taxa in the diet.

Arthropod Taxon	Virginia			Kentucky			West Virginia			TOTAL		
	N = 23			N = 15			N = 5			N = 43		
	Vol.	Freq.	No.	Vol.	Freq.	No.	Vol.	Freq.	No.	Vol.	Freq.	No.
Lepidoptera	58.5	95.7	22	80.3	100.0	15	90.0	100.0	5	70.0	97.7	42
Coleoptera (no ID)	13.2	65.2	15	6.0	60.1	9	1.0	40.0	2	9.5	61.0	26
No ID	6.2	34.8		1.3	26.7		0.4	20.0		3.8	27.9	
Scarabaeidae	5.3	21.7		3.0	26.7		0.6	20.0		4.2	25.6	
Chrysomelidae	1.7	8.7		1.7	6.7		0.0	0.0		1.5	7.5	
Diptera (no ID)	11.7	52.2	12	4.1	66.7	10	1.0	60.0	3	7.5	58.2	25
No ID	11.0	43.5		4.1	66.7		1.0	60.0		7.2	53.5	
Tipulidae	0.7	8.7		0.0	0.0		0.0	0.0		0.3	4.7	
Hymenoptera	1.7	4.3	1	0.4	13.4	2	0.4	20.0	1	1.2	9.3	4
Formicidae	1.7	4.3		0.3	6.7		0.4	20.0		1.1	7.0	
Ichneumonidae	0.0	0.0		0.1	6.7		0.0	0.0		0.1	2.3	

Neuroptera: Hemerobiidae	0.4	4.3	1	0.0	0.0	0	0.0	0.0	0	<0.0	2.3	1
Hemiptera	1.2	21.6	5	0.0	0.0	0	0.0	0.0	0	0.6	11.6	5
Pentatomidae	0.6	13.0		0.0	0.0		0.0	0.0		0.3	7.0	
Lygaeidae	0.2	4.3		0.0	0.0		0.0	0.0		0.1	2.3	
Reduviidae	0.4	4.3		0.0	0.0		0.0	0.0		0.2	2.3	
Homoptera: Cicadellidae	6.9	34.8	8	4.7	40.0	6	0.4	20.0	1	5.4	34.9	15
No ID insect	0.0	0.0		0.2	6.7		0.2	20.0		0.1	4.7	
Araneae	6.3	39.1	9	4.2	33.3	5	7.0	80.0	4	5.7	41.9	18
Total Occurrences			73			47			16			136
DDI			5.20			4.69			4.57			5.06

TABLE 2. Diet by percent volume and percent frequency are compared for states and total in the current and three similar studies. Taxa were ranked most to least common and for each taxon, rank for percent frequency is subtracted from rank for percent volume. This produces a zero when ranks are the same, or a positive or negative number, indicating the number or ranks percent volume is higher or lower (respectively) than percent frequency. When two or more taxa ranks are equal, comparisons between percent volume and percent frequency are shown with a slash (/).

	This study				Johnson et al.	Moosman et al.	Johnson and
	VA	KY	WV	Total	(2012)	(2007)	Gates (2007)
Lepidoptera	0	0	0	0	0	0	0
Coleoptera	0	-1	-1/0	0	0	0	0
Diptera	0	3	0/+1	0	0	0	0
Hymenoptera	-1/-2	0	-1	-1	0	0	0
Neuroptera	+1/0	0	0	0	1	0	0
Hemiptera	1	0	0	1	-1	1	0
Homoptera	-1	-1	-1	0	0	0	0
Orthoptera	0	0	0	0	0	0	0
Psocoptera	0	0	0	0	0	0	0
Trichoptera	0	0	0	0	0	0	0
No ID insect	0	0	+2/0	0	0	0	0
Araneae	1	-1	0	0	0	-1	0

TABLE 3. A comparison of arthropod prey in fecal samples by percent volume (Vol.) and percent frequency (Freq.), number of occurrences in the sample (No.), and diet diversity (DDI) in this and three similar studies.

	Current study			Johnson et al. (2012)			Moosman et al. (2007)			Johnson and Gates (2007)		
	N = 43			N = 77			N = 39			N = 44		
	Vol.	Freq.	No.	Vol.	Freq.	No.	Vol.	Freq.	No.	Vol.	Freq.	No.
Lepidoptera	70	97.7	42	41.8	100	77	46.2	85	33	58.5	98	43
Coleoptera	9.5	61.0	26	30.4	97	65	19.1	56	22	5.5	46	20
Diptera	7.5	58.2	25	16.7	82	63	18.6	46	18	24.7	66	29
Hymenoptera	1.2	9.3	4	0.7	9	7	0.1	3	1	5.4	27	12
Neuroptera	<0.0	2.3	1	2.3	32	25	0	0	0	1.6	7	3
Hemiptera[1]	0.6	11.6	5	3.3	29	22	5.2	21	8	1.0	5	2
Homoptera	5.4	34.9	15	0	0	0	<0.1	3	1	1.0	5	2
Orthoptera	0	0	0	0	0	0	0.1	3	1	0	0	0
Psocoptera	0	0	0	0	0	0	0	0	0	3.4	16	7
Trichoptera	0	0	0	1.8	16	12	2.0	8	3	0	0	0
Araneae	5.7	41.9	18	0.01	0.01	0	6.4	10	4	0	0	0
Occurrences			136			271			91			118
DDI			5.06			4.76			4.16			4.22

[1]Moosman et al. (2007) determined taxa of Hemiptera and Homoptera differently than the other studies

Comparison to Similar Studies

Prey by percent volume and percent frequency is similar among this and three previous studies (Table 3). ANOVA failed to identify a significant difference among studies by either percent volume ($F_{(3,40)} = .006$, $p > .99$) or percent frequency ($F_{(3,40)} = .255$, $p = .86$). Differences in ranking taxa by percent volume versus percent frequency among our combined sample and previous studies were infrequent, and never more than a single ranking (Table 2).

Diet diversity indices were also similar among studies. The DDI for this study was 5.06 and ranged from 4.16 to 4.76 for previous studies (Table 3). Because DDI were between four and five, the four most common taxa in the diet of our study were compared across studies by both percent volume and percent frequency (Fig.1). ANOVA failed to identify a significant difference among studies in the four common taxa by percent volume ($F_{(3,12)} = .036$, $p = .99$) or percent frequency ($F_{(3,12)} = .281$, $p = .84$).

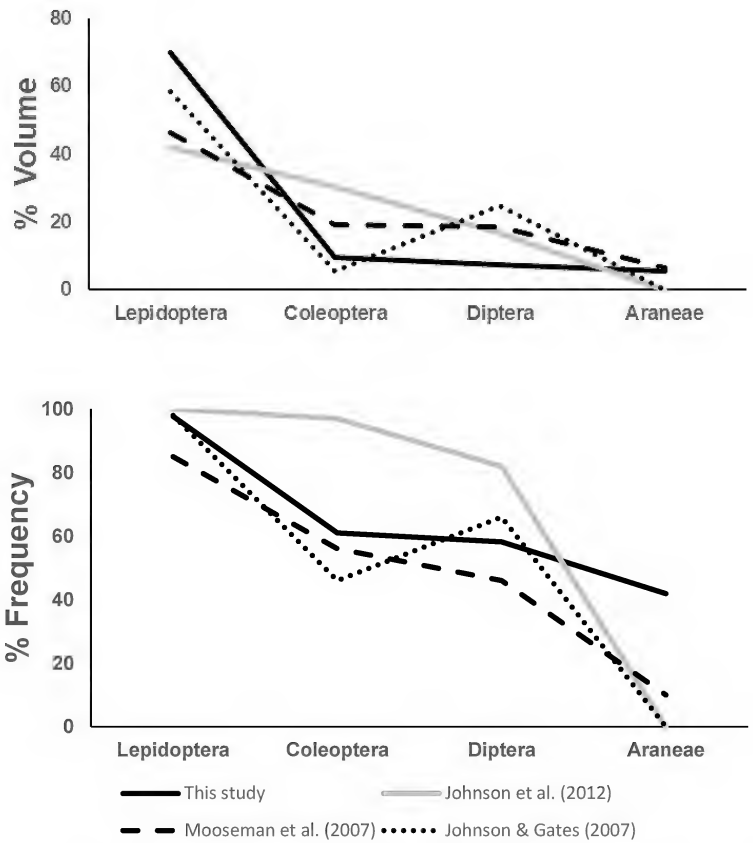

Fig. 1. A comparison of the four major taxa of arthropod prey identified in fecal samples from *M. leibii* in this and three similar studies by percent volume and percent frequency.

DISCUSSION

Summer fecal samples were obtained from bats netted in upland woods, typically with a mature oak hickory component, and prey eaten were more indicative of a terrestrial upland habitat than an aquatic habitat (Brack and LaVal 1985; Clare et al. 2011). It is not surprising that we did not detect differences among locations. Many species of insectivorous bats are generalists over time and space, and often, though not always, consume prey in general proportion to availability within habitats where they forage (Anthony and Kunz 1977; Brack and LaVal 1985, 2006; Sample and Whitmore 1993; Kurta and Whitaker 1998; Murray and Kurta 2002; Rakotoarivelo et al. 2007; Clare et al. 2011). Our data indicate *M. leibii* is a moth-beetle-fly-spider generalist, with an emphasis on moths. This bat flies slow and close (0.3 – 3 m) to the ground (Davis et al. 1965; Barbour and Davis 1969; Neuhauser 1971; van Zyll de Jong 1985), suggestive of a gleaning strategy.

Like past studies in western Maryland May–October (McDowell-Griffith 1983), New Hampshire May–September (Moosman et al. 2007), the Central Appalachians Ecoregion of southeastern West Virginia during autumn swarming (Johnson and Gates. 2007), and the Ridge and Valley Ecoregion in West Virginia spring–autumn (Johnson et al. 2012), the dominant food was moths. Similarly, in all these studies beetles and flies were second and third most common foods, although not always in the same order. Also, as in previous studies, insects from several additional orders comprised the remainder of the diet, although each contributed a small amount. Collectively, these data support the conclusion that this bat is a generalist, feeding in an upland, terrestrial-based habitat.

The DDI values were similar across areas of our study and across all studies. This index does not identify taxa consumed but does provide a comparison of a specialized or generalized diet. For example, DDI of *M. sodalis* and *M. grisescens* (Gray Myotis) both decrease from spring when food is least abundant to late summer when food is most abundant (Brack and LaVal 1985, 2006), and the diet of reproductive female *M. lucifugus* (Little Brown Myotis) is less diverse in summer (Anthony and Kunz 1977), when food is more abundant. Similarly, young less-skilled bats may have a lower DDI when concentrating on easily caught prey (Brack and LaVal 2006), and many species sharing a geographic area eat a common or superabundant prey (Storm and Whitaker 2008; Levin et al. 2009; Clare et al. 2011; Blazek et al. 2021), which can lower the DDI.

We report the highest percent frequency of spider consumption, total and in each state (33.3–80.0%), to date for *M leibii*, previously reported at 10.0% (Moosman et al. 2007) and 1.3% (Johnson et al. 2012). Bats may catch spiders by gleaning the surface of vegetation or the ground, or in the air where spiders are ballooning or are suspended on a web. The presence of crickets (family Gryllidae, order Orthoptera) in the diet (Moosman et al. 2007) supports the role of a gleaner. Many species of bats that are not gleaning specialists glean occasionally or under specific situations. For example, *M. lucifugus* is an aerial-hawing strategist (Anthony and Kunz 1977), but in Alaska 16.8% of the diet by volume is spiders (Whitaker and Lawhead 1992), in Ontario 7.6% of prey species identified using DNA is spiders (Clare et al. 2011), and in British Columbia, up to 8% of fecal samples contain spiders (Burles et al. 2008). Many species that glean also eat large quantities of moths. In western Indiana, spiders were in 25.4% and moths 69.8% of stomachs of

M. septentrionalis (Northern Long-eared Myotis) (Brack and Whitaker 2001), and in British Columbia > 70% of fecal samples from *M. keenii* (Keen's Myotis) contained moths and spiders. *Corynorhinus townsendii virginianus* (Virginia Big-eared Bat), another species that gleans and forages in Appalachian uplands, eats a high proportion of moths (Dalton et al. 1986; Sample and Whitmore 1993).

Based on standardized techniques (Whitaker 1988), many studies, including *M. leibii*, use percent volume and percent frequency to characterize consumption of taxa as a large proportion of diets versus widespread consumption among individuals. However, while comparison across the many taxa in a diet is difficult, ranking showed the two techniques provided similar results.

Our data support and broaden understanding of food habits of *M. leibii*, a species uncommon throughout its broad distribution. Across a broad geography and irrespective of season, sex, or age, this bat eats a relatively low-diversity diet centered on terrestrial-based insects, predominately moths, beetles, and flies. In addition, spiders are frequently consumed, which may reflect a greater use of a gleaning strategy than previously appreciated. These results highlight a need to better understand the foraging behavior and habitat of this bat.

A great deal remains to be learned about *M. leibii* and post-WNS (white-nose syndrome), it will be more difficult. WNS will likely forever change abundances and distributions of species of cave-hibernating bats in the eastern United States during winter (Frick et al. 2015; Powers et al. 2015) and summer (Dzal et al. 2010; Francl et al. 2012; Moosman et al. 2013; Reynolds et al. 2016). Species less impacted by WNS (Powers et al. 2016; Cheng et al. 2021) may have an advantage while loss or extreme rarity of a species may facilitate ecological release (Bolnick et al. 2010) of competitors, changing diets or habitats (Morningstar et al. 2019; Nocera et al. 2019; Mayberry et al. 2020). Changes resulting from WNS may allow species absent or at the periphery of their ranges, for example *M. grisescens*, to expand into the area (Powers et al. 2016), creating a new and different competition dynamic. These changes may make pre-WNS data helpful for understanding impacts of WNS and help put post-WNS studies in perspective.

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